Newborn screening for spinal muscular atrophy

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Clinical Features of Spinal Muscular Atrophy (SMA)

- A neuromuscular disease resulting in the progressive degeneration of motor neurons
- Symptoms include loss of normal motor function and respiratory failure; can result in death
- 3 clinical types based on age of onset and severity
  - Type I: Birth – 6 mos.
  - Type II: 6 mos. – 2 years
  - Type III: 18 mos. – 3+ years
SMA is the leading genetic cause of death among infants

- The birth prevalence of SMA is approx. 1 in 10,000
- Type I (infantile-onset) is the most common form
- The majority of children with Type I SMA do not survive beyond 2 years without effective therapy
- FDA approved therapy exists
- SMA has been nominated for inclusion on the Recommended Uniform Screening Panel (RUSP)
Genetic characterization of SMA

- Autosomal recessive inheritance
- Approximately 96% of SMA cases are caused by mutations in the \textit{SMN1} gene
  - \textit{SMN1} encodes for survival of motor neuron (SMN) protein
- Among the \textit{SMN1} mutations, most involve the loss of \textit{SMN1} exon 7 (on both chromosomes) by deletion or gene conversion
  - Loss of this gene region results in a non-functional SMN protein
  - \textit{SMN2}, a paralog of \textit{SMN1}, may moderate the disease severity
    - \textit{SMN2} can only produce 10% of the SMN protein produced by \textit{SMN1}

Several different molecular assays have been used to detect SMA

- Restriction Fragment Length Polymorphism (RFLP) test
- High Resolution Melting (HRM) analysis
- Multiplex Ligation-Dependent Probe Amplification (MLPA)
- Luminex Genotyping
- DNA sequencing
- Quantitative (qPCR)/ Real-time PCR (RT-PCR)
Real-time PCR is one method that can be used to screen newborns for SMA

- Real-time PCR allows for high throughput screening
- Most state newborn screening labs are already using this method
  - Labs are equipped with the necessary instrumentation
  - Staff is familiar with procedure
- Reactions can be multiplexed
  - Reduced cost to include a new assay
  - May not require added labor cost to run
What are some challenges associated with using real-time PCR to screen for SMA?
Challenge #1:

*SMN1* has a paralog, the *SMN2* gene, which has nearly identical genomic sequence

- There are only 5 nucleotide differences between the two genes
- For real-time PCR, it is important to avoid detecting *SMN2* when trying to identify the loss of *SMN1*
LNA (locked nucleic acid) nucleotides can be used to distinguish single nucleotide differences between \( SMN1 \) and \( SMN2 \). This would allow for discriminatory amplification and/or signal detection of \( SMN1 \) only.

- LNAs can be incorporated into primers and probes to discern single nucleotide differences between \( SMN1 \) and \( SMN2 \).

Initial SMA assay developed at CDC

**SMN1** (*SMN2*)  **Intron 7 Sequence**

```
ttttgtaaaaacttttatgtttttgtggaaaaacaaatgttttttgacatttaaaaagttcagatgttaA(G)aaagttgaaaggttaatgtaaaaaacatcaatattaagaattttgtgccc
```

- The loss of SMN1 intron 7 was detected using a LNA probe (in green)
  - LNA substitutions underlined
- The LNA probe was designed to selectively bind SMN1 by discriminating between the mismatch nucleotides of SMN1 and SMN2
  - SMN1 nucleotide (A) and SMN2 nucleotide (G)
- Forward and reverse primers (in yellow) will amplify both SMN1 and SMN2 sequences

Challenge #2: Recombination between SMN1 and SMN2 can result in a hybrid genotype

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SMN1

Exon 7 | Intron 7 | Exon 8

C  A

SMN2

Exon 7 | Intron 7 | Exon 8

T  G

False positive
8/120,000 (< 0.01%)*

False negative
Cases identified**; unknown prevalence

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We modified the previous assay to target exon 7 and reduce the possibility of false positive or false negative results due to hybrid genotypes.

- The LNA probe was designed to selectively bind \( SMN1 \) by discriminating between the mismatch nucleotides of \( SMN1 \) (C) and \( SMN2 \) (T).
- Forward and reverse primers (in yellow) will amplify both \( SMN1 \) and \( SMN2 \) sequences.

Assay gives *non-specific* amplification *some of the time* when testing samples derived from SMA patients.

- The LNA probe designed to recognize *SMN1* only can bind the *SMN2* amplicon, producing non-specific signal in SMA patient samples.
We replaced the original, reverse primer with an \textit{SMN1}-specific LNA primer (in blue) to eliminate \textit{SMN2} amplification.
Assay specificity improves by adding LNA primer

Assay Revision
Part 1

Assay Revision
Part 2

SMA patient samples

Non-specific signal from *SMN2*

No signal from *SMN2*
Technical concern:

Assay did not perform as expected in all environments

- Possible reasons for reduced assay efficiency:
  - Sensitive to DNA extraction method
  - Sensitive to type of Taqman master mix
  - Sensitive to temperature fluctuations > 1 degree Celsius

- Further method improvement was needed
LNA probe was redesigned to make the assay more robust

- Factors important in the design of LNA probe for mismatch discrimination:
  - Length of the probe
    - short (10-12 nucleotides)
  - Location of mismatch in the probe
    - center position within probe
  - Modification pattern
    - LNA substitution in triplet at site of mismatch
  - Identity of the mismatch
    - pyrimidine (C or T) at mismatch site within probe (discrimination is poor for G-T mismatches)

The Current Assay utilizes an $SMN1$-specific LNA probe with forward strand sequence.

- We do not observe any non-specific signal in $SMN1$ null samples even when challenged with an excess of $SMN2$ sequence.
This assay can also be multiplexed with primers and probes for RNase P ($RPP30$) and TREC.

Cq values for RNase P and TREC are unaffected by the addition of reagents for SMA.
The Current SMA Assay works at a range of temperatures from 60-65 degrees Celsius

- Patient samples are SMA test positive (no SMN1 signal) at temperatures ranging from 60-65 degrees Celsius
- Don’t need to worry about variations in instrument temperature affecting the results
The Current SMA Assay works at a range of temperatures from 60-65 degrees Celsius

- There is no observed effect of temperature on the Cq values for RNase P and TREC
- May not need to change the temperature of current TREC assay
- SMN1 amplification is not negatively affected
SMA patients are correctly identified from dried blood spots when using the current assay

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<th>Sample Number</th>
<th>Assay Results</th>
<th>Clinical Category</th>
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Key Features of the Current SMA assay

Assay Design Elements

- Targets Exon 7 and not Intron 7
- Forward Strand LNA Probe provides robust specificity (no background signal from SMN2)

Assay Characteristics

- Multiplex capable: can be used with TREC assay by adding only a few extra reagents; lower cost
- Sensitive: identified 100% of SMA patients with loss of SMN1 exon 7
- Flexible: (1) can be used at temperatures ranging from 60°C - 65°C, (2) works using “in situ” method and with DNA extracted from dried blood spots
Additional Key Points

- Both the LNA primer and forward strand (FS) probe improve specificity in detecting loss of \textit{SMN1} at exon 7.
- Current Assay using FS probe is comparatively more robust and cost effective.
- Use of a PCR clamp to suppress \textit{SMN2} amplification has also been developed, which can add an additional layer of specificity in a second tier assay for samples that are inconclusive.
- Droplet digital PCR can be used to determine copy number of \textit{SMN1} and \textit{SMN2}.
CDC can provide consultation and technical support to labs interested in screening for SMA

- **Pre assay development consultation**
  - Providing sequence for SMA assay primers and probe
  - Integrating SMA into current TREC assay

- **Reference materials for assay development and validation**

- **Individual training at CDC**
  - Performing real-time PCR assay
  - Preparation of QC materials
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Thank you for your attention!

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